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Introduction

To date it has been nearly impossible to distinguish whether the cellular transformation process gives rise to conditions that cause cancer cells to accumulate mutations (2), or whether an increase in the rate of mutation within cells gives rise to the transformation process that culminates in the formation of cancer cells (3,4). Currently, a strong case can be made relating the number of unrepaired mutations in the cell with the development of a cancerous phenotype (5-7). The mutations noted to correlate with the expression of a cancerous phenotype could result from an increase in errors made during both DNA replication and DNA repair (8-17). In order to begin to assess the degree to which errors created during DNA replication contribute to the overall mutation frequency observed in cancer cells, I proposed to compare the fidelity of the DNA replication process in malignant breast cells and normal breast cells. Several studies have reported that the activity of DNA β polymerase, an enzyme implicated in gap filling DNA synthesis during DNA repair (18,19) is decreased in cancer cells. These and other investigations also indicate that cancer cells generally have a higher error rate during the repair of gapped DNA and report that at least some of the common mutations include frame shifts and deletions of DNA sequence (17-22). However, most of these investigations were performed using either crude cell extracts or purified enzymes. *In vitro* assays using crude cell extracts contain nucleases and proteases, which may alter the integrity of the replication or repair enzymes in the extract or the DNA templates used in these assays. These factors may subsequently affect our interpretation of the data obtained using crude cell extracts. Those studies that use purified enzymes do not take into account that DNA repair in intact cells generally occurs in a highly controlled environment (13), with both the DNA and key enzymes organized into higher-order structures. These assays also do not adequately consider the potential contributions of accessory factors present in the intact cell that may enhance the fidelity of the DNA repair process. The observations reported by Kunkel's group (18,19) reinforce the idea that the maintenance of high fidelity DNA synthesis and repair requires at least some of the proteins used during DNA replication. Assays that ignore the possible involvement of the DNA replication proteins in the repair process are not capable of presenting an accurate picture of intact cell DNA repair, and also ignore the possible role played by the fidelity with which DNA replication is initially carried out and the overall contribution of the fidelity of DNA replication to the development of a "mutator" phenotype.

In order to better understand the extent to which the intact DNA replication machinery contributes to the overall mutation frequencies observed in normal and malignant breast cells, I have designed experiments to examine the degree of fidelity exhibited during the DNA replication process in both normal and cancerous breast cells.

To accomplish this goal I have isolated a multiprotein DNA replication complex (the MRC) from both normal breast tissue cells and malignant breast cancer cells and have begun to determine the ability of the MRC from both cell types to faithfully copy a target gene used in our *in vitro* replication assay system. We have previously shown that the MRC isolated from mammalian cells is fully competent to carry out large T-antigen-dependent DNA synthesis *in vitro* (23-25). The MRC has been purified to about 30-40 polypeptides and is fully competent to replicate DNA bidirectionally from a defined origin of DNA replication, producing semi-conservatively replicated DNA. The rate of DNA replication and the products of the *in vitro* reaction suggest that the MRC faithfully mimics the replication process carried out in intact cells. Using this multiprotein DNA replication complex we have initiated experiments intend to determine whether cancer cells exhibit a higher mutation frequency due to a defect in the fidelity of the DNA synthetic process. Initial data suggest that there is a higher frequency of mutation in our assay when the target DNA sequence is replicated by the MRC from the cancer cells than when it is replicated by the MRC from normal cells.

Materials and Methods

a. The pBK-CMV vector from Stratagene (see figure 3 in appendix) contains the full 298 bp SV40 origin of DNA replication, including both large T-antigen binding sites I and II. It also contains the eucaryotic promoter for the cytomegalovirus (CMV), the procaryotic RNA start sequence (at position 1221), the *lacP* gene (at position 1300-1220), which codes for the *lacZ* gene promoter, the α -*lacZ* gene (at position 1183-810), the start site for the β -gal gene (ATG) (at position 1183), and the stop site for the gene (TAA) (at position 799).

b. The XL1- Blue MRF' strain of *E. coli*: This strain was purchased from Stratagene as the optimal strain of *E. coli* for the growth and expression of the pBK-CMV vector. Electrocompetent XL1- Blue MRF' cells are purchased and transfected by electroporation (31,32).

c. Expression of the non-mutated β -galactosidase gene in the transformed *E. coli* growing in the presence of both the chromogenic substrate of the β -galactosidase gene product, 5-bromo-4-chloro-3-indolyl β -D galactoside (X-gal), and the inducer for the β -galactosidase gene, isopropylthio- β -D galactoside (IPTG), produces dark blue colonies. Errors in the sequence encoding the β -galactosidase gene result in white colonies.

Intermediate phenotypes (light blue) may result from less severe mutations of the gene encoding β -galactosidase.

Cell culture. The malignant breast cancer cell line, Hs578T (Homo sapiens No. 578, tumor cells), is an aneuploid, mammary epithelial cell line derived from a mammary tumor that does not express the estrogen receptor protein (26). The normal breast cell line Hs578Bst (Homo sapiens No. 578, breast cells) is diploid and is, most likely, of myoepithelial origin (26,34). It is derived from breast tissue found peripheral to the Hs578T tumor. Both cell lines were purchased from the ATCC. The Hs578T cells are grown in suspension in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L glucose, 10 units /ml bovine insulin, and 10% fetal bovine serum (FBS). The Hs578Bst cells are grown in monolayer culture with modified Dulbecco's medium, 30 ng/ml epidermal growth factor (EGF), and 10% FBS.

Fractionation scheme for the Isolation of Breast Cell Multiprotein DNA Replication Complex. Hs578T breast cancer cells (26) and Hs578Bst normal breast cells (26) are collected by low-speed centrifugation, and the cell pellet resuspended in PBS and frozen at -80°C using the procedure previously described by our laboratory (28,35,36). The human breast cell MRC are isolated using the procedure described by Malkas et al. (see figure 1 in appendix) (35). Briefly, frozen Hs578T and Hs578Bst cell pellets are thawed and resuspended in buffer, homogenized, and then centrifuged at 2,500 rpm ($1,740 \times g$) for 10 minutes in order to separate the crude nuclear (P-1) and cytosolic fractions (S-1). Mitochondria (P-2) are pelleted from the S-1 fraction by centrifugation at 12,500 rpm ($18,000 \times g$) for 15 minutes. The resultant supernatant (designated the S-2) fraction is then subjected to ultracentrifugation at $100,000 \times g$ for 1 hour to remove microsomes (P-3), and the supernatant are designated the S-3 fraction. The crude nuclear pellet (P-1) are resuspended in buffer and gently rocked for 2 hours. After a 10-minute centrifugation at $15,000 \times g$ the supernatant (designated NE), containing soluble protein extracted from the nuclei, is collected, combined with the S-3 fraction and made 2M in KCl and 5% in polyethylene glycol (PEG 6000). The mixture is stirred gently for one hour at 4°C and pelleted by centrifugation for 15 minutes at 16,000 rpm ($30,900 \times g$). The resultant supernatant (PEG NE/S-3) is collected and layered onto a 2M sucrose cushion and subjected to centrifugation at 40,000 rpm ($100,000 \times g$) for 16-18 hours at 4°C . The material above the sucrose cushion (top 80% of the tube) is collected and designated the S-4 fraction. The material collected at the sucrose interface is designated the P-4 fraction.

The P-4 fraction is then applied to a Q-Sepharose column (Pharmacia) (25 mg protein/1 ml of matrix) which is pre-equilibrated with loading buffer containing 50 mM Tris-HCl, pH 7.5 / 1 mM DTT / 1 mM EDTA / 10% glycerol / 50 mM KCl. Unbound protein is washed from the matrix with 8 volumes of column-loading buffer. The matrix-bound protein is eluted by an increasing KCl gradient (50 mM - 1 M) in 50 mM Tris-HCl, pH 7.5 / 1 mM DTT / 1 mM EDTA / 10% glycerol. The column fractions will then be assayed for their ability to support *in vitro* SV40 DNA replication. The column fractions able to support *in vitro* SV40 DNA replication are pooled and layered onto an 11-ml 10-30% sucrose gradient containing 50 mM Tris-HCl, pH 7.5 / 1 mM DTT / 1 mM EDTA / 0.5 M KCl. The tubes containing the sucrose gradients are centrifuged at 100,000 x g for 16 hours, and the sucrose gradient fraction containing the replication-competent MRC is aliquoted and stored at -80°C.

***In Vitro* DNA Replication Assay.** The reaction mixtures (50 μ l) contain 30 mM HEPES (pH 7.8), 7 mM MgCl₂, 0.5 mM DTT, 50 μ M dATP, 100 μ M each of dGTP, dCTP, dTTP, 5 μ Ci [α -³²P]dCTP (4,000 cpm/pmol), 200 mM each of CTP, UTP, GTP, 4 mM ATP, 40 mM phosphocreatine, 100 μ g of creatine phosphokinase, 15 mM sodium phosphate (pH 7.5), 30 ng of the plasmid pBK-CMV, and approximately 1.0 μ g of large T-antigen (3,23). The reaction mixture is incubated at 37°C for 3 hours. After 3 hours, 10 μ l of the reaction mixture is pipetted onto Whatman DE81 filters and allowed to air-dry. The filters are then washed 1 x (5 min.) with 0.1 M NaPPi buffer (pH 7.0), and 3 x (5 min.) with 0.3 M ammonium formate (pH 7.4), air-dried, and counted in a liquid scintillation counter.

Precipitation of the Replicated DNA. The DNA in the remaining 40 μ l from each *in vitro* DNA replication reaction are precipitated by the addition of ammonium acetate to 2.0 M (final concentration) and 2 volumes of 100% isopropanol. After centrifugation at 12,000 rpm (14,956 xg), the resulting pellets are dried and then each resuspended in 260 μ l TE buffer (10 mM Tris-HCl, pH 8.0 / 1 mM Na₄ EDTA). The DNA will then be further purified by phenol extraction (2 x 260 μ l) followed by diethyl ether (2 x 1.3 ml) extraction. The DNA are precipitated with 0.2 M Na Acetate and 70% ETOH. The precipitated DNA will then be pelleted by centrifugation, dried, resuspended in TE, and subjected to Dpn I digestion.

Analysis by Gel Electrophoresis of Replicated DNA Products. Approximately 2-3 μ l of the Dpn I-treated newly replicated DNA from each reaction mixture are mixed with 5 μ l of a sodium-dodecylsulfate (SDS)-containing dye mix (20 mM Tris-HCl, pH

8.0 / 5 mM EDTA / 5% SDS / 0.5% bromophenol blue / 25% glycerol). The pBK-CMV DNA are size fractionated by electrophoresis through a 0.8% agarose gel matrix at 40 V for 16 hours. The agarose gel matrix are prepared using Tris/glacial acetic acid/EDTA (TAE) buffer and 0.5 mg/ml ethidium bromide. The DNA products are visualized using autoradiography.

Forward Mutagenesis Assay: Transfection and Plating. (see figure 4 in appendix) The *in vitro* replicated pBK-CMV DNA from the Dpn I digestion will be used in the transfection of the *E. coli* host. Electrocompetent bacteria (*E. coli* strain XL1-Blue MRF') are mixed with approximately 300 pg of pBK-CMV DNA isolated from the *in vitro* DNA replication reaction, diluted to 40 μ l total volume with 10% glycerol in LB media, incubated for 10 min. on ice, and electroporated (1.4 kV, 25 μ F, 200 ohms). Immediately following the electroporation, 960 μ l of chilled sterile SOC buffer (20mM glucose in LB media) are added to the cuvette. The electroporated mixture is then incubated at 37°C for 1 hour at 250 rpm. An amount of the culture sufficient to yield 100-600 bacterial colonies per plate are plated onto 20 ml LB agar containing 0.5 mg/ml kanamycin, 25 mg/ml IPTG and 25 mg/ml X-gal. These plating conditions give intense blue color for the wild-type plasmid which facilitates the visualization of mutant phenotypes. The mutant colonies range from white to intermediate (relatively blue) phenotypes.

Scoring of Mutants. The inactivation of the α -complementation gene (the product of which is the catalytic subunit of β -galactosidase) due to a mutation in the *lacZ* α gene in pBK-CMV will give a variety of mutant phenotypes, due to the lack of a fully functional β -galactosidase gene product. These mutant phenotypes are scored after approximately 12-15 hours of incubation at 37°C. In order to reproducibly and precisely score the variable color intensity mutant phenotypes, a scale of blue color intensities has been established (1). Using the plating conditions described above, the wild-type pBK-CMV DNA generates a dark blue phenotype, which on a scale of 0-4, are assigned a value of 4. The variable mutant phenotypes can be distinguished as 0+ (white/colorless), 1+ (faint blue), 2+ (medium blue), or 3+ (almost wild type).

Since the background mutation rate for a *lacZ* α forward mutation assay employing M13 phage is typically about $2-5 \times 10^{-4}$, we have chosen a phagemid DNA, pBK-CMV, for which no detectable mutation rate has been demonstrated (Weeks and Hickey, unpublished data).

To eliminate other false positive mutants due to plating artifacts, mutant bacterial colonies are picked from the plates, diluted in 50 mM sodium borate buffer (pH 9.0) and

mixed with an equal dilution of the bacteria containing the unreplicated pBK-CMV plasmid. Plating of this mixture on the same plate enhances the contrast between the wild-type phenotype and the lighter blue mutants. It also permits more reproducible scoring of subtle phenotype differences arising from small variations in the position and number of point mutations within the *lacZ α* gene.

Determination of the nucleotide sequences of the DNA replicated *in vitro*. Single-stranded replicated DNA from the bacterial mutants selected as just described are isolated using the Quiagen Single-Stranded DNA isolation kit (each scored mutant phenotype separately) and sequenced by the dideoxynucleotide chain termination method as described by Sanger (29). Two individual oligonucleotides will prime the reactions for sequence analysis (see figure 5 in the appendix). The first is a 15-base primer complementary to the first 15 bp coding for the *lacZ α* gene. The second is a 15-base primer that codes for the last 15 bp of the *lacZ α* gene. Sequencing reactions using these two primers will span a region of 120 nucleotides (40 aa).

Results

I. Results of Task I : Preparation of the replication template DNA, months 1-12.

Since submitting the original proposal, in which we describe using the M13mp2 bacteriophage as the DNA template for the replication assay, we have discovered that M13mp2 bacteriophage has an intrinsic mutation rate of approximately $2-5 \times 10^{-4}$. In order to minimize the background mutation frequency rate we will use the pBK-CMV plasmid (from Stratagene), for which the detectable mutation rate has been observed to be less than 1×10^{10} colonies (Weeks and Hickey, unpublished data). As shown in figure 3, the pBK-CMV contains the SV40 origin of replication, including large T-antigen binding sites I and II, and the kanamycin resistance gene. We have successfully grown this plasmid in XL1-Blue MRF' E.coli, a strain selected for its ability to support optimal growth and expression of this plasmid (also from Stratagene), and isolated and purified the supercoiled form I plasmid DNA for use in the DNA replication assay.

II. Results of Task 2: Purification of the Multiprotein Replication Complex (MRC) , Months 1-12.

Our laboratory has isolated a multiprotein DNA replication complex (MRC) from human cervical carcinoma cells (HeLa) (35,36), from mouse mammary cells (FM3A)

(28) and most recently, from MDA MB 468 human breast cancer cells (36), and from a matched pair of human breast cell lines, Hs578Bst ("normal") and Hs578T (cancerous) (Weeks et al., unpublished data). The complex is isolated using a series of steps that includes ultracentrifugation, polyethylene glycol precipitation, and ion-exchange chromatography, as shown in the schematic figure 1. The sedimentation coefficient of the multiprotein complex from the MDA MB 468 breast cancer cells is approximately 18S as measured by sucrose gradient density analysis (36). The sedimentation coefficients of the Hs578Bst ("normal") and Hs578T (cancerous) cells MRCs are currently under analysis. The integrity of the multiprotein complex is maintained after treatment with DNase, RNase, 2 M KCl, NP40/butanol, and Triton X-100, and after chromatography on DE52-cellulose and Q-Sepharose, suggesting that the association of proteins with one another is independent of nonspecific interaction with other cellular macromolecular components (28).

Most importantly, we have demonstrated that the multiprotein replication complexes from the MDA MB 468 (36), and from the Hs578Bst and the Hs578T breast cell lines (Weeks et al., unpublished data), are fully competent to replicate DNA *in vitro* in a variation of the assay described by Li and Kelly (1984) (30). The demonstrated replication ability of the isolated multiprotein form of DNA polymerase suggests that all of the cellular activities required for large T-antigen-dependent *in vitro* papovavirus (i.e., SV40 and polyoma virus) DNA synthesis are present within the isolated DNA replication apparatus. Our lab has previously found that the mammalian MRC includes DNA polymerase α , DNA primase, DNA polymerase δ , proliferating cell nuclear antigen (PCNA), RP-A (a.k.a. RF-A, and HSSB), topoisomerases I and II, and RF-C or Activator 1 (Δ -1) protein complex (28,35,36). The presence of these enzymes in the MRC has been verified by both Western blotting and, when possible, enzyme activity assays (e.g., RP-A, RF-C, PCNA do not have intrinsic enzymatic activity). The most current model of the MRC is shown in figure 2 in the appendix.

III. Results of Task 3: Isolation and analysis of the MRC-mediated DNA replication products, months 1-12.

DNA replication products have been isolated and purified from Hs578Bst and Hs578T MRC-mediated *in vitro* replication assays. The purified DNA product has been subjected to Dpn I digestion and separated on a 1% neutral agarose gel. The products (visualized by autoradiography) demonstrate that the Hs578Bst and Hs578T MRC-mediated replication reactions are capable of producing a full length DNA replication product. The level of DNA replication in each reaction has also been

examined by measuring the incorporation of [^{32}P]- dCTP into the newly replicated DNA collected on DE81 filters and counted by liquid scintillation counting. Interestingly, the net nucleotide incorporation after a 3 hour incubation period is approximately 1.5 times higher in the replication reactions mediated by the cancerous Hs578T MRC than those mediated by the "normal" Hs578T MRC.

IV. Results of Task 4: Transfection and expression of the replicated DNA product in *E. coli*, months 12-36.

The XLI-Blue MRF' strain of *E. coli* was successfully transfected with each of the following : the wild type pBK-CMV, the fully replicated/Dpn I digested pBK-CMV, and an equimolar concentration of pUC19 DNA (negative control) as described in the methods. The entire electroporation mixture containing the transfected *E. coli* and SOC medium was plated (100 μl per plate) onto LB agar plates containing optimal concentrations of kanamycin, X-gal, and IPTG. The transfected *E. coli* containing the wild type pBK-CMV expressed a dark blue phenotype (100%). The *E. coli* transfected with the negative control DNA (pUC19) consistently created mutant (white) colonies (100%). The *E. coli* transfected with pBK-CMV replicated by the MRC from the Hs578T (cancerous) cell line demonstrated a mutant (white) phenotype with a frequency of 1.04 % ($x = 10.41/1000$ colonies). Mutant colonies created by the "normal" Hs578 Bst MRC-replicated DNA were only found with a frequency of 0.18% ($x = 1.84/1000$). To date, three separate experiments have been completed, under our empirically determined optimal conditions, all of which suggest that the frequency of replication errors in the Hs578T MRC-mediated replication reaction appears to be approximately 5.8 times higher than that of the plasmid replicated by the MRC from the "normal" Hs578Bst cells.

Mutant colonies were collected and grown separately in LB broth containing tetracyclin and kanamycin over night. The clonal populations of mutant *E. coli* were then collected by centrifugation, resuspended in fresh LB broth, made 10% in glycerol, and frozen at -80°C for later extraction and nucleotide sequencing of the mutant plasmid.

I am currently building a library of selected mutants, isolated from the blue/white mutant selection assay, in order to determine whether the mutations observed in the bacterial colonies isolated from the mutant selection assay occur randomly or are located to specific segments of the plasmid DNA template replicated by the breast cancer cell MRC.

Discussion

Our original proposal described experiments in which an M13 vector containing the SV40 viral origin of DNA replication and the β -galactosidase gene were covalently linked. This M13 vector was used in an *in vitro* DNA replication assay in which DNA synthesis was mediated by a multiprotein DNA replication complex (the MRC) isolated from both normal breast cells and breast cancer cells. Our goals were to determine whether the DNA synthetic machinery (the MRC) of breast cancer cells was more error-prone than the DNA synthetic machinery of normal breast cells, and whether our results supported the hypothesis that the higher incidence of mutations observed in breast cancer cells, was due to a reduction in the fidelity of the breast cancer cell MRC relative to the fidelity of the normal breast cell MRC.

In setting up the mutation selection assay, we discovered that M13 had an inherent mutation frequency of 2.5×10^{-4} . We believed that this high spontaneous rate of mutation, when the M13 was simply transfected into bacterial cells, would potentially mask the true mutation rate arising from errors created by the breast cancer cell MRC. If this were correct it would make it impossible to accurately assess whether the breast cancer cell MRC was error-prone. To overcome this potential difficulty we developed a strategy to covalently link the SV40 viral origin of replication and the β -galactosidase gene into a regular plasmid. During our initial planning of the details to construct this plasmid vector, we discovered that Stratagene had already constructed such a vector (pBK-CMV), and that it could be purchased from the company. We rapidly discovered that the Stratagene plasmid could be replicated *in vitro* by the MRC, and that the level of DNA replication could be optimized to that observed in the *in vitro* DNA replication assay employing another routinely used DNA template, pSVO+. The spontaneous mutation frequency of the unreplicated plasmid transfected into the XL1-Blue MRF' strain of *E. coli* was found to be less than 1×10^{-10} .

While this aspect of the project was being developed, our laboratory group isolated the multiprotein DNA replication complex from two matched human breast cell lines, Hs578BSt ("normal") and Hs578T (malignant) using the method previously described by our lab for other mammalian cell lines (27,28,35, 36). The MRC from each of these two human breast cell lines is fully competent to complete full length, semiconservative, large T-antigen dependent *in vitro* DNA replication. This fact, as well as results described previously by our lab (27,28,35,36) suggest that all of the cellular protein activities necessary for *in vitro* SV40 DNA synthesis are present within the MRC isolated from the Hs578Bst and the Hs578T human breast cells. Additionally, the requirements for SV40 DNA replication *in vitro* by the isolated human breast cell MRC are comparable to the

requirements that have been observed with crude cell extracts from permissive cells (30); namely, the initiation of SV40 DNA synthesis is dependent on the presence of both large T-antigen and a functional SV40 replication origin sequence.

An initial assessment of the level and fidelity of DNA replication carried out by the MRC of each of the two human breast cell lines suggests a direct relationship between the relative rate with which the DNA template is replicated by the MRC and the relative frequency of mutational sequence errors that are created in the replicated DNA. Thus far, we have observed the level of DNA replication to be 1.5 times higher in the replication reactions mediated by the breast cancer cell MRC than in those reactions mediated by the normal breast cell MRC. The apparent increased rate of replication carried out by the breast cancer cell MRC correlates with an increased (5.8 times) frequency of mutant (white) colonies observed in the bacteria transfected with the plasmid replicated by the breast cancer cell MRC than in the bacteria transfected with plasmid replicated by normal breast cell MRC. Although, to date, we cannot yet describe the actual differences, in the breast cancer cell MRC and the normal breast cell MRC, responsible for the altered replication rate and replication fidelity of the breast cancer cell MRC, it is likely that alteration in specific components of the MRC are responsible for these differences.

I have just begun to establish the DNA sequencing methodology needed to analyze the nucleotide sequence of the defective β -galactosidase genes being collected from the mutant bacterial colonies selected in our assay. In addition, I am expanding my initial analysis of the fidelity of the breast cancer cell MRC and the normal breast cell MRC in order to develop an expanded pool of bacterial mutants expressing defects in the β -galactosidase gene. This pool of mutants will permit us to determine whether the defects in the β -galactosidase gene occur randomly or are localized to specific segments of the gene. This type of information, such as the appearance of mutation types characteristic of errors in proof-reading, will increase our understanding of the types of changes which may distinguish the cancerous breast cell MRC from the normal breast cell MRC.

To date, our results support the hypothesis that the cellular transformation process is, at least partially, a consequence of an increased rate in the accumulation of mutations and that these mutations arise, in part, due to a decrease in the fidelity of the DNA replication machinery (i.e. MRC) of the cancer cell.

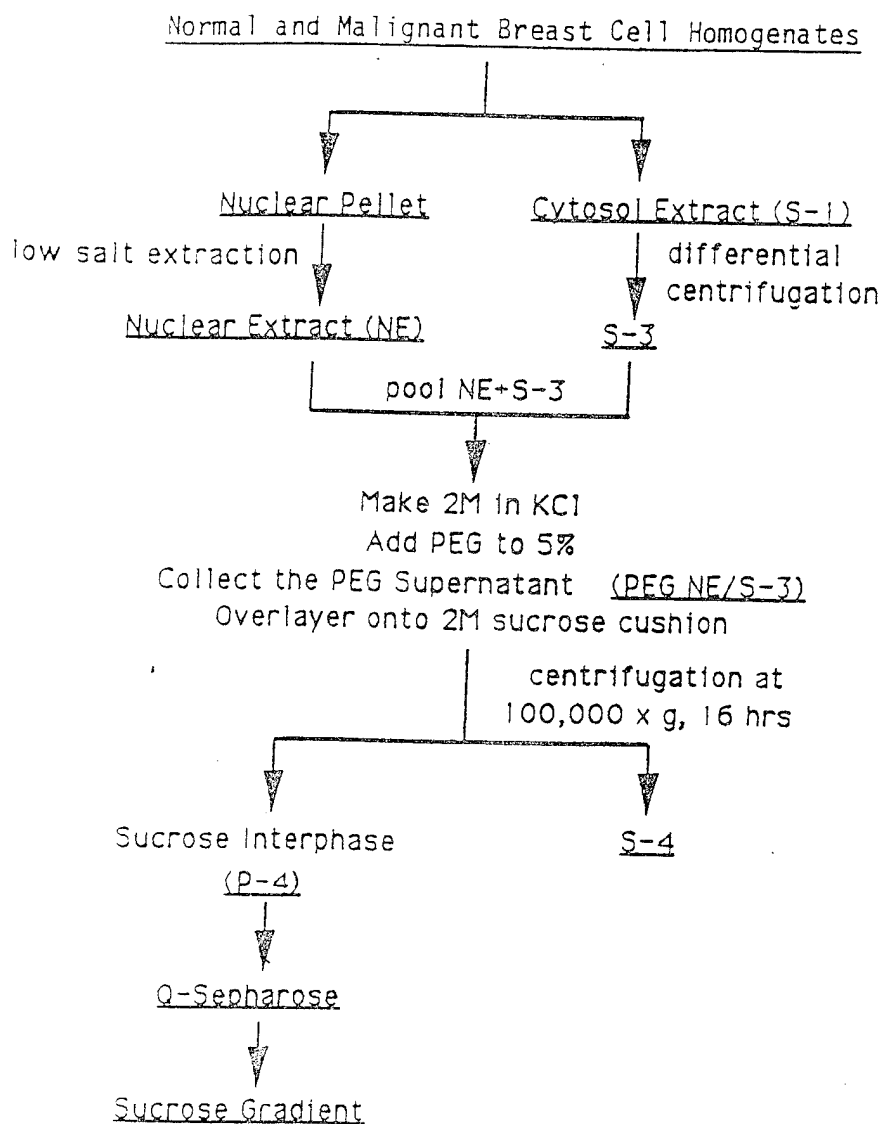
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Current Model for the MRC

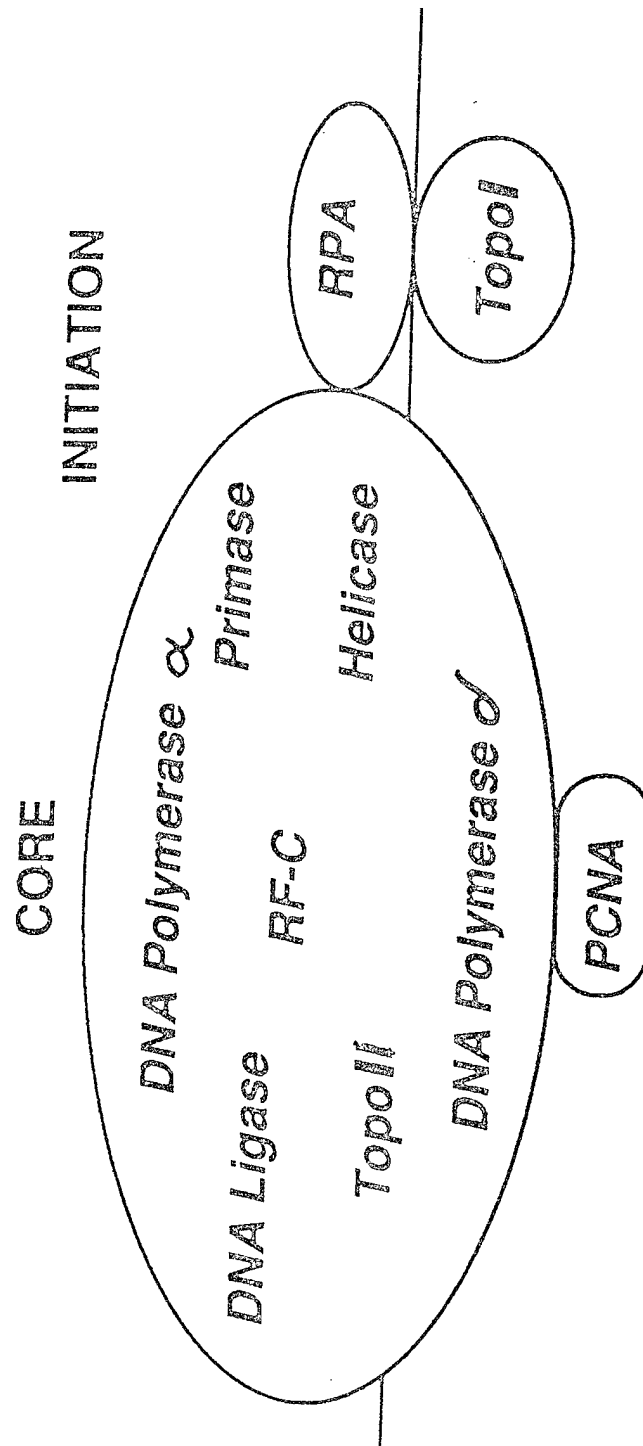
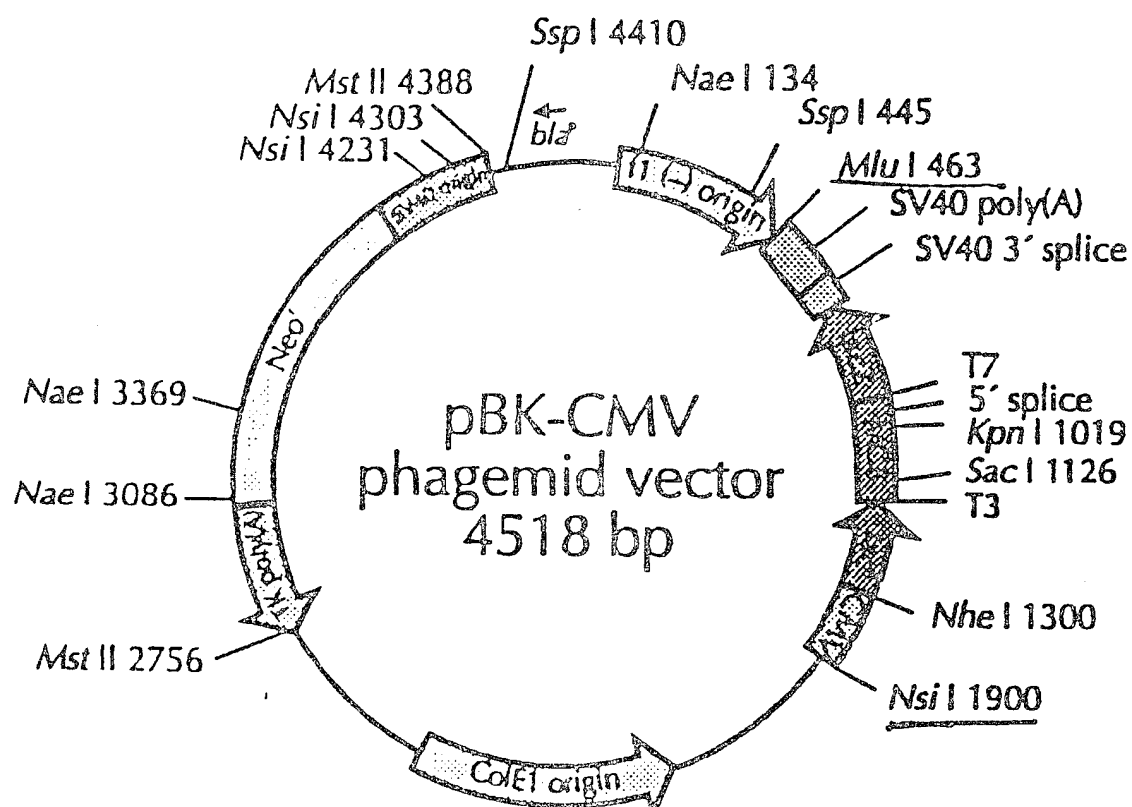
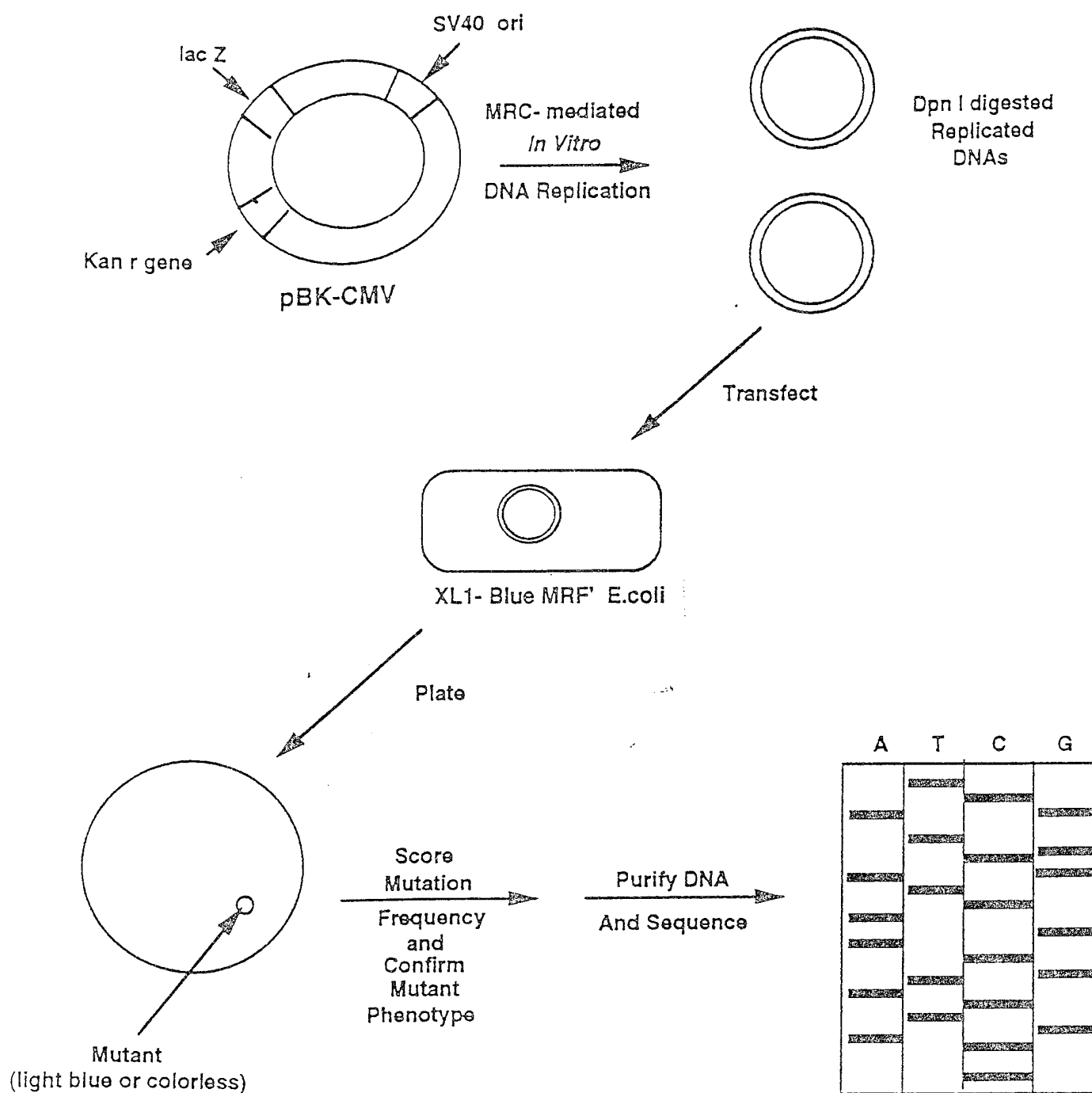


Figure 3



FORWARD MUTAGENESIS ASSAY: PART I

Sequencing primers for the α -lacZ gene

Primer 1

810

TC TCCATTTCGCC ATTCAGGCTG CGCAACTGTT GGGAAGGGCG
ATCGGTGCGG GCCTCTTCGC TATTACGCCA GCTGGCGAAA GGGGGATGTG

901

CTGCAAGGCG ATTAAGTTGG GTAACGCCAG GGTTTTCCCA GTCACGACGT
TGTA AACGA CGGCCAGTGA ATTGTAATAC GACTCACTAT AGGGCGAATT

1002

GGGTACACTT ACCTGGTACC CCACCCGGGT GGAAAATCGA TGGGCCCCGCG
GCCGCTCTAG AAGTACTCTC GAGAAGCTTT

1081

TTGAATTCTT TGGATCCACT AGTGTCGACC TGCAGGCGCG CGAGCTCCAG
CTTTTGTTCC CTTTAGTGAG GGTTAATTTC GAGCTTGGCGAATCAAGGTCA

Primer 2